PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

(MBHB Case No. 02-1049-B)

In the Application of:	
Jenny et al.	**Confirmation No. 5272**
Serial No.: 10/534,187	**Examiner: Susan Marie Hanley**
Filed: November 9, 2005	**Group Art Unit: 1651**
For: Assay For Tissue Factor and Factor VIIa	

DECLARATION OF RICHARD JENNY PURSUANT TO 37 C.F.R § 1.131

I, Richard Jenny, hereby declare:

1. I am a named co-inventor on U.S. Patent Application Serial No. 10/534,187, filed on November 9, 2005. The other named co-inventor is Saulius Butenas.

2. The invention disclosed and claimed in the instant patent application was first conceived in the United States before November 5, 2002, the earliest possible effective filing date for the relied-upon subject matter in U.S. Patent No. 7,049,087, naming Richard Jenny, Paul E. Haley, and Brian Earp as inventors.

3. Accompanying this Declaration as Exhibit A is a photocopy of a Grant Application that I submitted to the U.S. Department of Health and Human Service on July 31, 2002, which is before the earliest possible effective filing date for the relied-upon subject matter in U.S. Patent No. 7,049,087.

4. Exhibit A (see, for example, pages 13-16) is directed to the invention claimed in my U.S. Patent Application Serial No. 10/534,187 and evidences both my conception and reduction to practice of my invention at least as early as July 31, 2002, which is before

November 5, 2002, the earliest possible effective filing date for the relied-upon subject matter in U.S. Patent No. 7,049,087.

5. U.S. Patent Application Serial No. 10/534,187 claims priority from three priority documents. The first is U.S. Provisional Application No. 60/425,662, filed November 13, 2002; the second is U.S. Provisional Application No. 60/466,214, filed April 28, 2003; the third is PCT/US2003/35608, filed November 6, 2003.

6. I was diligent from a date prior to July 31, 2002 (the date Exhibit A was submitted to the U.S. Department of Health and Human Service), to November 13, 2002, the date of my legal constructive reduction to practice, *i.e.*, the filing of U.S. Provisional Application No. 06/425,662. With the assistance of my patent attorney, Steve Sarussi, I prepared several revised drafts in August to November of 2002. The final one was filed with the U.S. Patent and Trademark Application on November 13, 2002 and which was assigned U.S. Provisional Application No. 06/425,662.

7. I hereby declare further that all statements made herein to my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 12/22/2008

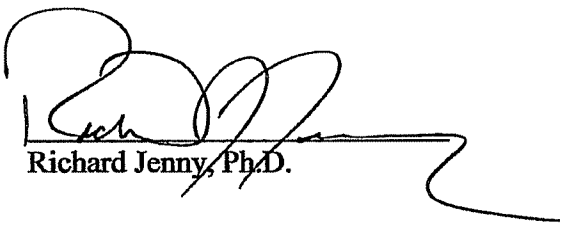
Signed: 
Richard Jenny, Ph.D.

Exhibit A

Form Approved Through 05/2004 OMB No. 0925-0001

Department of Health and Human Services
Public Health Services

Grant Application

Do not exceed 56-character length restrictions, including spaces.

LEAVE BLANK—FOR PHS USE ONLY.		
Type	Activity	Number
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT
Fluorogenic Assays for Factor VIIa and Tissue Factor

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR — New Investigator ☒ No ☐ Yes

3a. NAME *(Last, first, middle)*: Jenny, Richard J.

3b. DEGREE(S): B.S. Ph.D

3c. POSITION TITLE: President and Scientific Director

3d. MAILING ADDRESS *(Street, city, state, zip code)*:
Haematologic Technologies, Inc.
57 River Road
Unit 1021
Essex Junction, Vermont
05452

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT:

3f. MAJOR SUBDIVISION:

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: (802) 878-1777 FAX: (802) 878-1776

E-MAIL ADDRESS: rjenny@haemtech.net

4. HUMAN SUBJECTS RESEARCH ☒ No ☐ Yes
4a. Research Exempt ☐ No ☐ Yes — If "Yes," Exemption No.
4b. Human Subjects Assurance No.
4c. NIH-defined Phase III Clinical Trial ☐ No ☐ Yes

5. VERTEBRATE ANIMALS ☒ No ☐ Yes
5a. If "Yes," IACUC approval Date
5b. Animal welfare assurance no

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
03/01/03	08/31/03	$75,000	$75,000	$75,000	$75,000

9. APPLICANT ORGANIZATION
Name: Haematologic Technologies, Inc.
Address: 57 River Road
Unit 1021
Essex Junction, Vermont
05452

Institutional Profile File Number (if known)

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☐ Private Nonprofit
For-profit: → ☐ General ☒ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
03-0312741
DUNS NO. *(if available)* 192057123
Congressional District: Vermont at Large

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name: Richard J. Jenny, Ph.D.
Title: President and Scientific Director
Address: Haematologic Technologies, Inc.
57 River Road, Unit 1021
Essex Junction, Vermont 05452
Tel (802) 878-1777 FAX (802) 878-1776
E-Mail rjenny@haemtech.net

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: Richard J. Jenny, Ph.D.
Title: President and Scientific Director
Address: Haematologic Technologies, Inc.
57 River Road, Unit 1021
Essex Junction, Vermont 05452
Tel (802) 878-1777 FAX (802) 878-1776
E-Mail rjenny@haemtech.net

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)* — DATE: 07/31/02

15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)* — DATE: 07/31/02

DESCRIPTION:[JG2] State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Factor VIIa and Tissue Factor (TF) are essential proteins for the initiation of blood coagulation. Blood coagualtion is initiated when cryptic TF becomes exposed on the surface of vascular cells where it can bind circulating factor VIIa. The factor VIIa/TF complex catalyzes the activation of certain blood zymogens that propogate the coagulation event. The amount of circulating factor VIIa has been shown to be a good indicator of hemostatic potential and for this reason is a potential risk indicator for the development of cardiovascular disease. In addition, over the past decade recombinant factor VIIa has become the drug of choice for treating hemophilia A and B patients who have developed inhibitors to factors VIII and IX respectively. Formation of the factor VIIa/TF complex is also the basis of specific coagualtion assays. The Prothrombin time (PT assay) utilizes either natural or synthetic thromboplastin reagents to initiate coagulatoin in-vitro. Thus in addition to its in-vivo role, TF also has in-vitro applications. Furthermore, TF is not only present in the vasculature, but also in a numerous other tissues and cells including brain, lung, placenta, platelets, monocytes and tumor cells. In addition to its "normal" role in hemostasis, it is also known to be involved in the metastasis of tumor cells. The fact that TF and factor VIIa play such important roles both in-vivo and in-vitro, indicates that rapid and direct assays for these proteins could be of great utility. At the present time, reliable assays for factor VIIa and tissue factor that can be applied to simple as well as complex biologial systems do not exist. The goal of this phase I proposal is to demonstrate the feasibility of developing flurogenic-based assays that may be used to directly measure factor VIIa and TF in simple and complex biological systems. To accomplish this goal, the following specific aims are proposed: 1) To utilize aminonaphthalene-based fluorogenic substrates for factor VIIa to develop basic assays that may be used to quantitate factor VIIa and TF in simple and well defined systems; b) to further develop the assay for tissue factor and demonstrate the ability to measure TF in more complex biological systems; and c) to further develop the assay for factor VIIa and demonstrate the ability to measure factor VIIa in more complex biological systems.

PERFORMANCE SITE(S) *(organization, city, state)*
Haematologic Technologies, Inc.
Essex Junction, Vermont

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
Richard J. Jenny, Ph.D.	Haematologic Technologies, Inc.	Principal Investigator
Meghan Anderson	Haematologic Technologies, Inc.	Technician
Amanda Driscoll	Haematologic Technologies, Inc.	Technician
Saulius Butenas, Ph.D.	University of Vermont	Consultant
Kenneth G. Mann, Ph.D.	University of Vermont	Consultant

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☒ **Yes** ☐ **No**

The name of the principal investigator/program director must be provided at the top of each printed page and each continuation page.[JG3]

RESEARCH GRANT
TABLE OF CONTENTS

Appendix *(Five collated sets. No page numbering necessary for Appendix.)*

Check if Appendix is Included ☐

Appendices NOT PERMITTED for Phase I SBIR/STTR unless specifically solicited.

Number of publications and manuscripts accepted for publication *(not to exceed 10)* _____

Other items (list):

BUDGET JUSTIFICATION PAGE MODULAR RESEARCH GRANT APPLICATION				
Initial Budget Period	Second Year of Support	Third Year of Support	Fourth Year of Support	Fifth Year of Support
$ 75,000	$	$	$	$
Total Direct Costs Requested for Entire Project Period				$ 75,000

Personnel

Richard J. Jenny, Ph.D. (HTI)	Principal Investigator	25% Effort
Meghan Anderson (HTI)	Technician	100% Effort
Amanda Perkins (HTI)	Technicnan	20% Effort
Saulius Butenas (University of Vermont)	Consultant	< 5% Effort
Kenneth G. Mann, Ph.D. (University of Vermont)	Consultant	<5% Effort

Consortium

Fee (SBIR/STTR Only)

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Richard J. Jenny, Ph.D.	President and Scientific Director

EDUCATION/TRAINING *(Begin. with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Wisconsin, Eau Claire, Wisconsin	B.S.	1980	Chemistry
University of Vermont, Burlington, Vermont	Ph.D.	1989	Biochemistry

A. Positions and Honors.

PROFESSIONAL ORGANIZATIONS:

American Heart Association: Council on Thrombosis, Member
American Association for the Advancement of Science, Member

RESEARCH EXPERIENCE:

1979 – 1980	Laboratory Assistant, Dept. of Chemistry, University of Wisconsin, Eau Claire, Wiscon
1981 - 1984	Laboratory Research Technician, Hematology Research, Mayo Clinic, Rochester, Minnesota
1984 - 1989	Laboratory Research Technician, Dept. of Biochemistry, Univ. of Vermont, Burlington, Vermont
1986 - 1989	Graduate Student, Dept. of Biochemistry, University of Vermont, Burlington, Vermont
1989	Post Doctoral Fellow, Dept. of Biochemistry, University of Vermont, Burlington, Vermont
1989 - present	President and Scientific Director, Haematologic Technologies, Inc., Essex Junction, Vermont

AWARDS AND HONORS:

XIth International Congress on Thrombosis and Hemostasis Award in Recognition for Outstanding Research Communication, July 1987.

JOURNALS

Member, Editorial Board, BIOTECHNOLOGY AND APPLIED BIOCHEMISTRY(1995-present)

B. Selected peer-reviewed publications (in chronological order).

PUBLICATIONS:

1) Tracy, R.P., Young, D.S., Katzman, J.A. and Jenny, R.J.: Two-dimensional gel electrophoresis in the systematic development of new laboratory tests. Ass. NY Acad. Sci. 428: 144-157, 1984.
2) Litwiller, R.D., Jenny, R.J., Katzman, J.A., Miller, R.S. and Mann, K.G.: Monoclonal antibodies to human vitamin K-dependent protein S. Blood 67: 1583-1590, 1986.
3) Jenny, R.J., Church, W.R., Odegaard, B.H., Litwiller, R.D. and Mann, K.G.: Purification of six human vitamin K-dependent proteins in a single chromatographic step using immunoaffinity columns. Preparative Biochemistry 16: 227-245, 1986.
4) Litwiller, R.D., Jenny, R.J. and Mann, K.G.: Identification and isolation of vitamin K-dependent proteins by HPLC. Analytical Biochemistry 158: 355-360, 1986.
5) Jenny, R.J., Pittman. D.D., Toole, J.J., Kriz, R.W., Aldape, R.A., Hewick, R.M., Kaufman, R.J. and Mann, K.G.: Complete cDNA and derived amino acid sequence of human factor V. Proc. Natl. Acad. Sci. USA 84: 4846-4850, 1987.

6) Mann, K.G., Tracy, P.B., Krishnaswamy, S., Jenny, R.J., Odegaard, B.H. and Nesheim, M.E.: Platelets and Coagulation. In Thrombosis and Haemostasis 1987 (Verstraete, M., Vermylen, J., Lijnen, R., Arnout, J., eds.). Leuven University Press, Leuven, Belgium (1987), pp. 505-523.
7) Nesheim, M.E., Abbott, T., Jenny, R. and Mann, K.G.: Evidence that the thrombin-catalyzed feedback cleavage of fragment 1.2 at Arg154-Ser155 promotes the release of thrombin from the catalytic surface during the activation of prothrombin. J. Biol. Chem. 263: 1037-1044, 1988.
8) Gross, M.D., Simon, A.M., Jenny, R.J., Gray, E.D., McGuire, D.M., Van-Pilsum, J.F.: Multiple forms of rat kidney L-arginine:glycine amidinotransferase. J. of Nutr. 118: 1403-1409, 1988.
9) Mann, K.G., Jenny, R.J. and Krishnaswamy, S.: Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. Ann. Rev. Biochem. 57: 915-956, 1988.
10) Jenny, R.J., and Mann, K.G.: Factor V: A prototype pro-cofactor for vitamin K-dependent enzyme complexes in blood clotting. In Bailliere's Clinical Haematology, Vol 2, No. 4, (1989), pp. 919-944.
11) Kalafatis, M., Jenny, R.J., and Mann, K.G.: Identification and characterization of a phospholipid-binding site of bovine factor Va. J. Biol. Chem. 265: 21580-21589, 1990.
12) Kalafatis, M., Rand, M.D., Jenny, R.J., and Mann, K.G.: Phosphorylation of factor Va and factor VIIIa by activated platelets. Blood 81(3): 704-719, 1993.
13) Jenny, R.J., Messier, T.L., Ouellette, L.A., and Church, W.R.: Immunochemical Techniques for Studying Coagulation Proteins. In Methods in Enzymology, Vol. 222, 400-416, 1993.
14) Tracy, R.P., Jenny, R.J., Williams, E.B., and Mann, K.G.: Active Site-Specific Assays for Enzymes of Coagulation and Fibrinolytic Pathways. In Methods in Enzymology, Vol. 222, 514-525, 1993.
15) Jenny, R.J., Tracy, P.T., and Mann, K.G.: Physiology and Biochemistry of Factor V. In Haemostasis and Thrombosis, 3rd Edition, Vol. 1, pp. 465-476. (Bloom A.L., Forbes C.D., Thomas D.P., Tuddenham E.G.D., eds.), Churchill Livingston, London, 1994.
16) Bovill, E.G., Tracy, R.P., Hayes, T.E., Jenny, R.J., Bhushan, F.H., and Mann, K.G.: Evidence that Meizothrombin is an Intermediate Product in the Clotting of Whole Blood. Arterosclerosis, Thrombosis and Vascular Biology 15: 754-758, 1995.
17) Lundblad, R.L., Bergstrom, J., De Vreker, R., Bray, G., Gomperts, E., Baker, D., Kingdon, H.S., Mann, K.G., Hartshorn, J., and Jenny, R.J.: Measurement of Active Coagulation Factors in Autoplex®-T with Colorimetric Active Site-Specific Assay Technology. Thromb. Haemost. 80:811-815, 1998.
18) Taatjes, D.J., Quinn, A.S., Jenny, R.J., Haley, P.H., Bovill, E.G., and McDonagh, J.: Tertiary Structure of the Hepatic Cell Protein Fibrinogen in Fluid Revealed by Atomic Force Microscopy. Cell Biology Int. 21: 715-726, 1998.

ABSTRACTS:

1) Litwiller, R.D., Jenny, R.J., and Mann, K.G.: Monoclonal Antibodies to Human Protein S. American Society of Hematology, 26th Annual Meeting, 1984.
2) Jenny, R.J., Ehrlich, Y.H., Korneki, E. and Mann, K.G.: Kinase mediated phosphorylation of factor V/Va. Circulation 74: 1645, 1986.
3) Ehrlich, Y.H., Korneki, E., Jenny, R., Cierniewski, C.S. and Mann, K.G.: Regulation of platelet activation and blood coagulation by extracellular protein phosphorylation systems. Blood 68: Suppl. 1, 315a, 1986c.
4) Jenny, R.J., Pittman, D.D., Toole, J.J., Kriz, R.W., Kaufman, R.J. and Mann, K.G.: The complete amino acid sequence of human factor V. Thrombosis and Haemostasis 58(1): 298, 1987.
5) Jenny, R.J. and Mann, K.G.: Auto-phosphorylation of the factor Va heavy chain. Blood 74: Suppl. 1, 353a, 1989.
6) Howard, P. Jenny, R.J., Church, W. and Tracy, R.: Development of a human factor VII ELISA: Preliminary studies.
7) Kalafatis, M., Jenny, R.J., and Mann, K.G.: A Membrane Binding Fragment of Bovine Factor Va. Circulation 82(4): 129, #511, 1990.
8) Pittman, D.D., Tompkinson, K., Marquette, K., Jenny, R.J., Mann, K.G., and Kaufmann, R.J.: Expresion of Recombinant Factor V and B-Domain Mutants in Mammalian Cells. Blood 76(10): 433a, #1722, 1990.
9) Bovill, E.G., Tracy, R.P., Hayes, T., Jenny, R.J., Bhushan, F., and Mann, K.G.: Meizothrombin is an Important Intermediate Product in the Conversion of Prothrombin to Thrombin During Whole Blood Clotting. XIV Congress of the International Society of Thrombosis and Haemostasis. New York, July 4-8, 1993.

10) Bray, G.L., Kingdon, H., Lundblad, R., DeVreker, R., Gomperts, E., Baker, D., Bergstrom, J., Mann, K., Hartshorn, J. and Jenny, R.J.: Characterization of Active Coagulant Enzymes in Autoplex®T. Blood 86(10): 195a, #766, 1995.

C. Research Support.

NONE

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Saulius Butenas, Ph.D.	Research Associate Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Technological University of Kaunas, Kaunas, Lithuania	B.S.	1972	
Institute of Biochemistry of Lithuanian Academy of Sciences, Vilnius, Lithuania	Ph.D.	1985	Organic Chemistry

A. Positions and Honors

1978-1985 Junior Scientist, Institute of Biochemistry, Plunge, Lithuania
1986-1987 Scientist, Institute of Enzymology, Vinius, Lithuania
1988-1990 Research Fellow, Institute of Molecular Genetics, Moscow, USSR
1989-1991 Senior Scientist, Institute of Biochemistry, Vilnius, Lithuania
1991-1997 Postdoctoral Associate, Department of Biochemistry, University of Vermont, Burlington, VT.
1997-Present Research Associate Professor, Department of Biochemistry, University of Vermont, Burlington, VT.

B. Selected peer-reviewed publications (in chronological order).

1. Lawson JH, **Butenas S**, Mann KG. The evaluation of complex dependent alterations in human factor VIIa. *J Biol Chem* **267**, 4834-4843 (1992).
2. **Butenas S**, Orfeo T, Lawson JH, Mann KG. Aminonaphthalenesulfonamides, a new class of modifiable fluorescent detecting groups and their use in substrate for serine protease enzymes. *Biochemistry* **31**, 5399-5411 (1992).
3. Talaikite Z, **Butenas S**, Palaima A. Substituted 6-amino-1-naphthalenesulfonamides as fluorogenic leaving groups of synthetic protease substrates. *Bioorg Khim* **18**, 828-836 (1992).
4. Lawson JH, **Butenas S**, Ribarik N, Mann KG. Complex-dependent inhibition of factor VIIa by antithrombin III and heparin. *J Biol Chem* **268**, 767-770 (1993).
5. Lawson JH, Krishnaswamy S, **Butenas S**, Mann KG. Proteolytic activity of the extrinsic pathway. *Methods Enzymol* **222**, 177-194 (1993).
6. **Butenas S**, Ribarik N, Mann KG. Synthetic substrates for human factor VIIa and factor VIIa-tissue factor. *Biochemistry* **32**, 6531-6538 (1993).
7. **Butenas S**, Lawson JH, Kalafatis M, Mann KG. Cooperative interaction of divalent metal ions, substrate, and tissue factor with factor VIIa. *Biochemistry* **33**, 3449-3456 (1994).
8. **Butenas S**, Drungilaite V, Mann KG. Fluorogenic substrates for activated protein C. Substrate structure-efficiency correlation. *Anal Biochem* **225**, 231-241 (1995).
9. **Butenas S**, Mann KG. Kinetics of human factor VII activation. *Biochemistry* **35**, 1904-1910 (1996).
10. **Butenas S**, Kalafatis M, Mann KG. Analysis of tissue plasminogen activator specificity using peptidyl fluorogenic substrates. *Biochemistry* **36**, 2123-2131 (1997).
11. **Butenas S**, van 't Veer C, Mann K G. Evaluation of the initiation phase of blood coagulation using ultrasensitive assays for serine proteases. *J Biol Chem* **272**, 21527-21533 (1997).
12. **Butenas S**, DiLorenzo M, Mann KG. Ultrasensitive fluorogenic substrates for serine proteases. *Thromb Haemost* **78**, 1193-1201 (1997).
13. Mann KG, van 't Veer C, Cawthern K, **Butenas S**. The role of the tissue factor pathway in initiation of coagulation. *Blood Coagul Fibrinolysis* **9, Suppl. 1**, 53-57 (1998).
14. van 't Veer C, **Butenas S**, Golden NJ, Mann KG. Regulation of prothrombinase activity by protein S. *Thromb Haemost* **82**, 80-87 (1999).

15. Brummel KE, **Butenas S**, Mann KG. An integrated study of fibrinogen during blood coagulation. *J Biol Chem* **274**, 22862-22870 (1999).
16. **Butenas S**, van 't Veer C, Mann KG. "Normal" thrombin generation. *Blood* **94**, 2169-2178 (1999).
17. **Butenas S**, van 't Veer C, Cawthern K, Brummel KE, Mann KG. Models of blood coagulation. *Blood Coagul Fibrinolysis* **9 (Suppl. 1)**, S9-S13 (2000).
18. Undas A, Williams EB, **Butenas S**, Orfeo T, Mann KG. Homocysteine inhibits inactivation of factor Va by activated protein C. *J Biol Chem* **276**, 4389-4397 (2001).
19. **Butenas S**, Cawthern KM, van 't Veer C, DiLorenzo M, Lock JB, Mann KG. Antiplatelet agents in tissue factor-induced blood coagulation. *Blood* **97**, 2314-2322 (2001).
20. **Butenas S**, Branda RF, van 't Veer C, Cawthern KM, Mann KG. Platelets and phospholipids in tissue factor-initiated thrombin generation. *Thromb Haemost* **86**, 660-667 (2001).
21. Butenas S, Brummel KE, Branda RF, Paradis SG, Mann KG. Mechanism of factor VIIa-dependent coagulation in hemophilia blood. *Blood* 99(3), 923-930 (2002).
22. Butenas S, Mann KG. Blood coagulation. *Biochemistry* (Mosc) 67(1),3-12. Review (2002).
23. Brummel KE, Paradis SG, Butenas S, Mann KG. Thrombin functions during tissue factor-induced blood coagulation. *Blood*. 2002 100(1):148-52 (2002).

C. Research Support.

BUTENAS, S.
ONGOING

2 P01 HL46703-11 Mann (PI) 9/1/01 – 8/31/06
NHLBI
Surface Dependent Reactions in Thrombosis & Thrombolysis

Project 1: "Activation of Prothrombin"
The major goals of this project are 1) To quantitate protein-protein reactions involved in prothrombinase and other vitamin K-dependent protein complexes; 2) evaluate tissue factor/factor VIIa complex; 3) quantitatively evaluate thrombomodulin interactions with Xa and other proteases; 4) evaluate reactions product formation during combined vitamin K-dependent complex reactions; 5) pursue development of TIRF technology as a tool for studying surface dependent reactions.

PHS R01 HL34575-17 Mann (PI) 1/5/99-12/31/03
NHLBI
Primary Structure of Prothrombin

The major goals of this project are objective concerns structural studies on Factor V and Factor VIII, the plasma procofactors for the prothrombinase and tenase complexes. This includes peptide chemistry and molecular biology studies of Factor V and Factor VIII. Another aim involves further studies on abnormal prothrombins and identification of specific sequence defects.

Baxter Hyland 06/20/00-06/19/02
Elevated AT-III and PC or Vitamin K-Dependent Protein Levels and Thrombin Generation

COMPLETED

2 P01 HL46703-10 Mann (PI) 9/1/96 – 8/31/01
NHLBI
Surface Dependent Reactions in Thrombosis & Thrombolysis

Project 1: "Activation of Prothrombin"
The major goals of this project are 1) To quantitate protein-protein reactions involved in prothrombinase and other vitamin K-dependent protein complexes; 2) evaluate tissue factor/factor VIIa complex; 3) quantitatively evaluate thrombomodulin interactions with Xa and other proteases; 4) evaluate reactions product formation during combined vitamin K-dependent complex reactions; 5) pursue development of TIRF technology as a tool for studying surface dependent reactions.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:
HTI maintains a 10,000 square foot facility in Essex Junction, Vermont. This new facility, which was completed in July of 2000, replaced the former 5500 square foot facility that HTI had previously occupied. The facility includes 7500 square feet of laboratory space.
The laboratory is designed specifically to handle process scale protein purification, protein characterization, and contract research in the areas of thrombosis and hemostasis. For cold workspace and storage purposes the laboratory houses three walk-in cold rooms (4°C), fifteen standard laboratory freezers (-20°C), two critical temperature freezers (-30°C) and three ultra-cold freezers (-70°C).

Clinical:

Animal:

Computer:
HTI operates it's own network system with 15 workstations, one main server and a mail server.

Office:
The facility includes 2500 square feet of office space. The office area consists of a main office/reception suite, three private offices, eight semi-private office cubicles, a conference room, a full kitchen and breakroom, two bathrooms (M/F), a library, and an office machine room.

Other:

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Major equipment includes: five coagulation analyzers, one clinical chemistry analyzer, seven floor model centrifuges, three bench-top centrifuges, three automated chromatography instruments (AKTA explorer, FPLC), two HPLCs, two spectrophotometers, one pulsed-beam fluorometer, two conventional ELISA plate readers, a fluorescent plate reader, a submersible-probe sonicator, an automated ELISA plate coater, and automated ELISA plate reader, two fume hoods, one laminar-flow hood, a lyophilizer, and a central RO water system equipped with two high-volume satellite deionizing stations. In addition to the major equipment, there are multiple pH meters, balances, stir-plates, overhead stirrers, vortexers, etc. Major safety equipment includes: two overhead showers, one drench hose, three eyewash stations, two chemical spill stations, and multiple fire extinguishers.

A) SPECIFIC AIMS

The primary goal for this phase-I proposal is to demonstrate the feasibility of producing ultrasensitive fluorogenic-based assays for factor VIIa and TF in a high-throughput microtiter plate format. The assays will be essentially identical in design and will rely on the ability of factor VIIa to hydrolyze a synthetic fluorogenic substrate at a rate that is TF dependent. In the assay for factor VIIa, the concentration of factor VIIa will be varied while the concentration of TF will be held fixed at a saturating concentration. The opposite will be done for the TF assay, with the TF concentration varied and the factor VIIa concentration fixed. The assays would ultimately be utilized for the quantitation of these proteins in purified systems as well as in complex biological mixtures such as plasma, cells, cell homogenates and tissue extracts. To accomplish this goal, the following specific aims are proposed:

Specific Aim #1:

Basic assay parameters and limitations will be established for the assay of factor VIIa and Tissue Factor by varying assay conditions while using purified proteins in well defined buffer systems. We will explore the limitations of the microtiter plate format with regard to sensitivity. In the process of doing so we will evaluate the effect of using different microtiter plates, different ANSN-based fluorogenic substrates for factor VIIa/TF, and various buffer conditions with emphasis on detergent concentrations and the presence or absence of calcium. We will vary enzyme, cofactor and substrate concentrations to establish optimal conditions. We will utilize both detergent-solubilized TF and relipidated TF to establish our limitations with regard to tissue factor presentation.

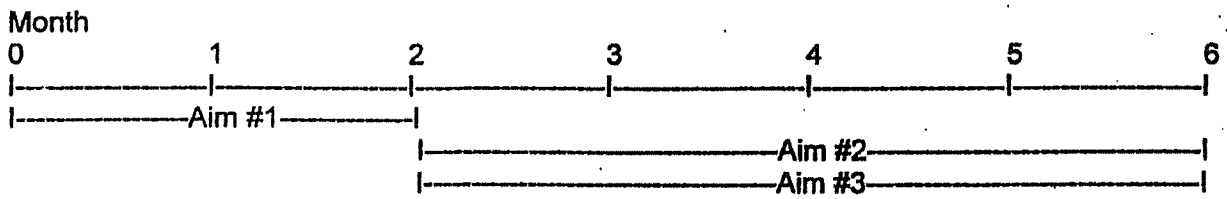
Specific Aim #2:

The assay for TF will be applied to more complex biological systems including: thromboplastin reagents (both synthetic and natural), tissue/cell homogenates or extracts and plasma. We will examine different sample preparation techniques including organic and/or detergent extractions. We will evaluate a direct assay approach as well as an immuno-capture method. The direct approach will involve placing the samples directly into the assay well along with the fluorogenic substrate and a fixed amount of factor VIIa. The immuno-capture method will involve capturing the TF into assay wells that have been coated with anti-TF antibodies, then washing away extraneous components leaving only the captured TF. Factor VIIa would then be added and following a brief incubation, substrate would be added to initiate the assay. It is anticipated that the immuno-capture method would alleviate any problems that could arise due to the complex sample matrix. We will perform recovery studies using assay samples that have been spiked with purified antigen to demonstrate the specificity of the assay.

Specific Aim #3:

The assay for factor VIIa will be applied to more complex biological systems with particular emphasis on plasma. For factor VIIa, we will also examine a direct assay approach as well as an immuno-capture method. Plasma assays will be done in the presence of EDTA to prevent further activation of endogenous factor VII and other serine proteases. We will perform the assays in the presence and absence of TF to assure that the enzyme activity that we measure is factor VIIa. If necessary, we will experiment with the use of specific protease inhibitors to minimize background activity from other serine proteases. We will perform recovery studies using assay samples that have been spiked with purified antigen to demonstrate the specificity of the assay.

Timeline: We anticipate being able to accomplish the specific aims according to the following time line:



B) BACKGROUND AND SIGNIFICANCE

Tissue factor (TF) and factor VIIa are essential components for the initiation of blood coagulation. The blood coagulation cascade is initiated when cryptic TF is expressed/exposed to the circulating blood where it can bind circulating factor VIIa. The resulting factor VIIa-TF complex activates the zymogens factor IX and factor X to the respective enzymes factor IXa and factor Xa. The factor Xa initially produced activates a small amount of prothrombin to yield picomolar amounts of thrombin, which in turn activates platelets and the procofactors factors V and VIII. Activated factor VIII (factor VIIIa) forms a complex on a membrane surface with serine protease factor IXa and activates factor X at a 50-100-fold higher rate than the factor VIIa-TF complex. The factor Xa produced, in complex with the cofactor, factor Va, and an appropriate membrane surface forms the prothrombinase complex, which is the primary activator of prothrombin. The thrombin produced amplifies its own generation by activating factor XI to factor XIa (which can activate more factor IX) and completes the activation of platelets and procofactors. The thrombin that is generated cleaves fibrinogen to form fibrin and also activates factor XIII to factor XIIIa. Factor XIIIa is a transglutaminase that catalyzes the cross-linking of fibrin to form the insoluble isopeptide cross-linked fibrin clot[1].

A number of studies have demonstrated relationships between circulating factor VIIa levels and hemostatic potential. It has been established that hemophilia A and B patients have a decreased level of circulating factor VIIa[2], whereas elevated levels of this enzyme are observed in patients with cardiovascular diseases[3-5]. Furthermore, in recent years recombinant factor VIIa has been successfully used for the treatment of hemophilia in patients with and without inhibitors[6-10], and most commonly in those hemophiliac patients undergoing surgical procedures[8-10]. Moreover, recombinant factor VIIa has been suggested as a treatment for almost any bleeding diathesis or for the enhancement of normal hemostasis in patients without coagulation defects[11].

The mechanism of the establishment of hemostasis by high doses of factor VIIa is not clear, and the regimen of patient treatment is usually selected by mimicking previously reported successful ones. However, even in case of the same bleeding disorder, an identical treatment regimen does not guarantee an identical outcome. For example, in one particular study only four out of eight hemophilia patients with inhibitors achieved normal hemostasis even though all of them received "standard" injections of 90 μg/kg factor VIIa[12]. On the other end of the spectrum, an attempt to use the same treatment regimen for a different bleeding diathesis could result in an

overdose of factor VIIa and, as a consequence, a risk of thrombosis. Another consideration with regard to the use and dosage of recombinant factor VIIa is the fact that it is extremely expensive and excessive use adds unnecessary costs to patient treatment. With total treatment regimens using up to several grams of material, the total cost for recombinant factor VIIa can run into the hundreds of thousands of dollars[13,14]. The ability to monitor a patients factor VIIa levels would likely translate to the use of less material and thus less cost per patient. Collectively, these observations lead to the conclusion that an individualized regimen has to be developed for every type of bleeding disorder and, desirably, for every patient as well.

A reliable and relatively simple assay for factor VIIa concentration in plasma is a necessary diagnostic tool. It would aid in the design of individualized treatment regimens and could be used for monitoring the hemostatic potential during periods of patient evaluation, treatment and post-treatment. In addition, because of the relationship between elevated factor VIIa levels and cardiovascular disease, the assay may have applications as a screening tool to discern an individuals risk of developing cardiovascular disease.

Although several assays for plasma factor VIIa have been described (some of which are commercially available), the majority of those assays do not discriminate between factor VII and factor VIIa, either due to the lack of specificity in immunologic methods[15,16], or due to the feedback-activation of factor VII in the amidolytic and clot-based assays[17-20]. Less than a decade ago, a "direct" factor VIIa assay in plasma was described based upon clotting of plasma initiated with a soluble mutant of TF[21]. This mutant TF is not able to promote factor VII activation and therefore has led the developers of the assay to claim that factor VIIa concentration remains unaltered during the assay. However, it is established that the most potent activator of factor VII in plasma is factor Xa and not the factor VIIa/TF complex [22]. Several studies also suggest that factor IXa is a major in vivo activator of factor VII[2,23]. Thus, it is obvious that concentration of factor VIIa in plasma must increase during the assay due to the back-activation of factor VII. Additionally, a clotting assay that involves entire coagulation cascade is sensitive to the concentration of procoagulant proteins and coagulation inhibitors. As a consequence, the clotting time of plasma initiated with soluble TF reflects not only the concentration of factor VIIa but also the concentration of all components of plasma involved in coagulation and its regulation. This conclusion is supported in part by a published study showing dependence of factor VIIa assay on protein C deficiency[24].

A new class of highly sensitive fluorogenic substrates for serine proteases that contain 6-amino-1-naphthalenesulfonamide (ANSN) leaving groups have been described[25,26]. These substrates are useful for the quantitation of serine proteases of coagulation and fibrinolysis at picomolar concentrations [27,28] (Table 1). Several substrates that are useful for the quantitation of factor VIIa yield nearly a 100-fold increase in the rate of hydrolysis when TF is added to form the factor VIIa/TF complex. These substrates allow for a direct TF-dependent quantitation of factor VIIa and sensitivity into the low picomolar range [21,28-28]. Additionally, the amidolytic activity of the factor VIIa/TF complex toward peptidyl-ANSN substrates in the presence of EDTA is comparable with that observed in the presence of divalent metal ions[29] (Figure 1). These properties of ANSN substrates lead to the assumption that: a) a direct quantitation of picomolar concentrations of factor VIIa can be done; b) assays can be done in the presence of EDTA thus providing a way to prevent activation of the zymogens of blood coagulation present in plasma (including factor VII); c) the presence of other enzymes should not interfere with factor VIIa quantitation due to the specific response of this enzyme to the presence of TF. In addition, interfering enzymatic activity that may be present in plasma and which is not related to factor VIIa, can be inhibited by selective inhibitors (hirudin for thrombin, tick anticoagulant peptide for factor Xa, etc.)

Table 1. Quantitation limits (QL) of serine proteases.

Substrate	Enzyme	K_M μM	k_{cat} s^{-1}	k_{cat}/K_M $M^{-1}s^{-1}x10^{-5}$	Quantitation		
					[S] μM	Incub. time min	QL pM
D-AFK-ANSNH(i-C_4H_9)[a]	Plm	110	3.3	0.3	100	60	1
D-VPR-ANSNH(n-C_4H_9)	FIIa	2.0	114	570	50	15	0.02
D-VPR-ANSNH(n-C_4H_9)	APC	54	72	13	50	20	0.8
Boc-D-VPR-ANSNH(n-C_4H_9)[b]	tc-uPA	95	4.0	0.4	100	60	1
D-FPR-ANSNH(n-C_4H_9)	FVIIa	190	0.11	0.006	100	120	40
D-FPR-ANSNH(n-C_4H_9)	FVIIa/TF	100	2.7	0.3	100	120	1
D-LPR-ANSNH(n-C_3H_7)	FXIa	75	53	7.1	100	60	0.01
Mes-D-LGR-ANSN(C_2H_5)$_2$[c]	Fxa	120	36	2.9	100	20	0.4

[a]i-C_4H_9-isobutyl;[b]Boc-tert-butoxy-carbonyl;[c]Mes-methanesulfonyl

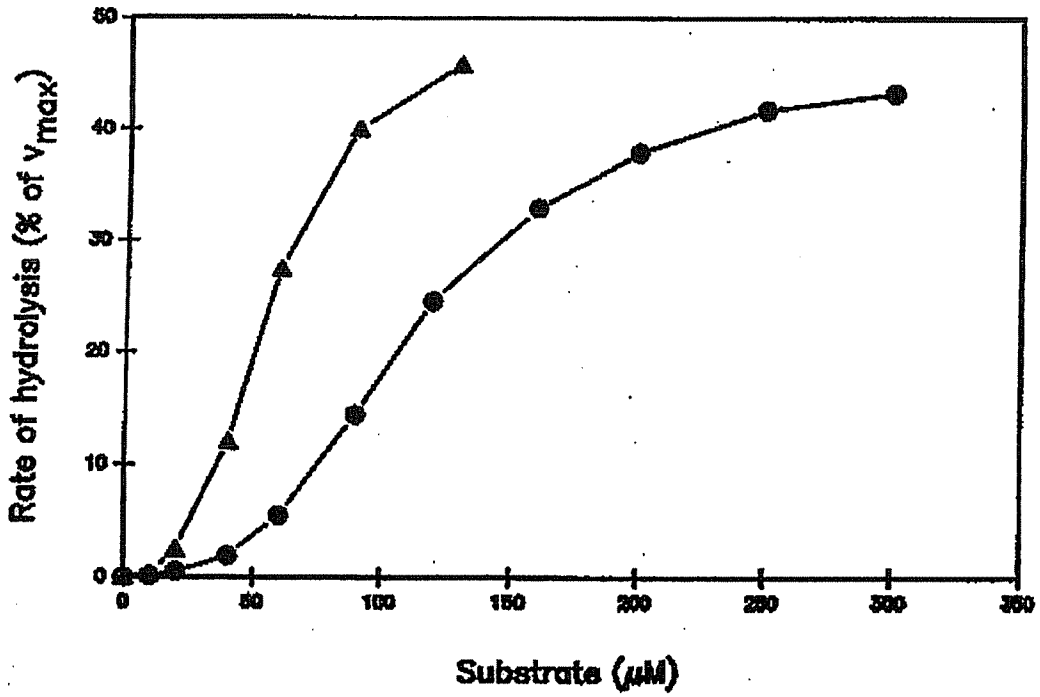


FIGURE 1: Peptidyl-ANSN substrate hydrolysis by factor VIIa in the presence of TF and EDTA. Factor VIIa, TF and EDTA were preincubated in HBS at 37°C for 10 min. Increasing concentrations of the substrate D-FPR-(propyl)ANSN (▲) or the substrate D-FPR-(cyclohexyl)ANSN (●) were added and the initial rates of substrate hydrolysis were evaluated. The fact that the factor VIIa assay is TF dependent suggests that the same assay could be re-engineered to yield an assay specific for TF. TF is an integral membrane protein that is

expressed in a variety of different cell types. It is expressed in the vascular adventitia, in astroglial cells, in organ capsules, etc., and is found in the central nervous system, lungs, and placenta at relatively high concentrations[30]. Monocytes and macrophages are known to express TF [31-33] and recently, several laboratories published data indicating that TF may be present in platelets and in fact may circulate in blood, although the existence and role of such TF remains controversial [34-37]. In addition to the expression in normal tissues and cells, it is also known to be expressed by tumor cells where it is related to the metastatic potential of those cells[38]. Furthermore, TF has been identified in atherosclerotic plaques[39], which suggests a role for TF in the progression of atherosclerotic cardiovascular disease.

Although it may appear somewhat ubiquitous, the primary known function of TF is the initiation of blood coagulation. This occurs when cryptic tissue factor is expressed/exposed on the surface of vascular cells where it can bind factor VIIa and form the TF/factor VIIa complex. In addition to being important from an *in-vivo* hemostatic perspective, the formation of the factor VIIa/TF complex is the basis for one of the most commonly utilized coagulation assays, the Prothrombin Time (PT assay). The PT assay is used routinely as a way to quantify hemostatic potential. It has many applications, but is most commonly used to screen pre-operative patients and to monitor and gage the administration of anticoagulant therapies. The assay involves the addition of a highly characterized thromboplastin reagent to citrated plasma samples and simultaneous or subsequent recalcification. The time required for clot formation following recalcification is the PT time. Variations of this assay can be performed with factor deficient plasmas to create PT-based assays that are specific for prothrombin, factor V, factor VII, and factor X.

Thromboplastin reagents are produced and sold commercially by numerous manufacturers. The reagents can be made by homogenizing natural tissues that contain a relative abundance of TF such as brain, placenta and lung, or in an approach that is becoming more common, can be made synthetically by incorporating recombinant TF into synthetic phospholipid vesicles using a relipidation procedure. The variety of different thromboplastin reagents that became available created a problem with regard to standardization of the PT assay. Because each type of PT reagent can yield a different clot time for the same plasma sample, a method of standardization was urgently needed. As a result, the concept of an INR (International Normalized Ratio) was developed. The INR is a value that is obtained by dividing the patient PT by the laboratory "normal" PT and raising the product to the "ISI" power. The ISI (International Sensitivity Index) is a number that is established by the manufacturer for each lot of thromboplastin that is produced. The number is derived by comparison of the manufacturers material to the WHO (World Health Organiztion) standard thromboplastin in a test that is instrument specific. The WHO thromboplastin standard has an ISI value of 1.0, and in an ideal world, all manufacturers would produce a thromboplastin with an ISI value of 1.0. The challenge of attaining this goal is related to assuring that the proper ratio of functional TF to phospholipid is attained in the final PT reagent. At the heart of this problem is the inability to accurately measure functional TF.

Due to the critical role that TF plays in hemostasis, its potential role in metastasis as well as its extensive use in-vitro, it is important to have a sensitive and specific TF assay able to detected relatively low amounts of this protein in biological fluids, cell cultures, lysates, as well as in purified and semi-purified systems. TF assays that have thus far been developed employ clotting[36,40], chromogenic[41] and immunochemical[37] methods. The clotting methods for assaying TF involve the entire coagulation cascade and are therefore sensitive to the alterations in the levels of procoagulant proteins and coagulation inhibitors. Chromogenic methods do not allow a direct measure of TF activity and are expensive, since they require additional purified

coagulation factors. Similarly, immunochemical methods are relatively expensive as well as time-consuming. Thus at the present time, a quick, accurate and somewhat universal method to directly measure TF activity does not exist.

A functional-based assay that could be used to measure TF in purified and/or complex biological systems would have a variety of potential applications incuding: a) in-vitro diagnostics for the assessment of hemostatic potential; b) in-vitro diagnostics for thrombotic risk assessment; c) in-vitro diagnostics for cancer screening; d) quality control during the purification of recombinant tissue factor; e) quality control during the manufacture of PT reagents; and f) characterization of final TF and PT reagents.

It has been demonstrated that the amidolytic activity of the TF/factor VIIa complex toward small fluorogenic substrates is membrane (phospholipid) independent[42]. This observation suggests that TF can be successfully quantitated in a free form in purified systems and biological fluids as well as present on cell or artificial membranes and in cell lysates. Fluorogenic substrates, which allow a quantitation of low concentrations of factor VIIa (as described above), will similarly allow the quantitation of similarly low concentrations of TF.

C. PRELIMINARY DATA

The 6-amino-1-naphthalenesulfonamide (ANSN) family of fluorogenic substrates and their utility have been described in numerous research publications and in U.S. patent number 5,399,487, which has been licensed and assigned to Haematologic Technologies, Inc. on an exclusive basis. These substrates have been used in variety of experimental procedures to monitor TF dependent factor VIIa activity. It has been demonstrated that the properties of these substrates will allow for the direct quantitation of factor VIIa at low picomolar concentrations in purified systems. More recently the use of these substrates for the development of ultrasensitive assays that can be applied to more complex biological systems has been investigated.

With regard to factor VIIa, we have used one of the peptidyl-ANSN substrates, particularly D-FPR-(cyclohexyl)ANSN, in an attempt to perform direct factor VIIa assay in plasma. Initially, varying concentrations of factor VIIa (0-1000 pM) were incubated with 5 nM TF in 20 mM Hepes, 0.15 M NaCl, pH 7.4 (HBS), containing 20 mM EDTA for 10 min at room temperature, followed by the addition of 100 µM D-FPR-(cyclohexyl)ANSN. Substrate hydrolysis was monitored continuously for 5 min in a fluorometer at an excitation wavelength of 350 nm and an emission wavelength of 470 nm. Rates of hydrolysis were determined for each factor VIIa concentration tested and a calibration line was constructed (Figure 2). The factor VIIa/TF-dependent rate of substrate hydrolysis was linear over the range of factor VIIa concentrations tested. It should be noted that the rate of substrate hydrolysis in the absence of tissue factor is about 100-fold less and is therefore negligible.

In a second step, normal citrated plasma was diluted (1:1) with HBS containing 20 mM EDTA, and the pH was adjusted to 7.4. Next, 5 nM TF was added and the plasma was incubated for 10 min at room temperature followed by the addition of 100 µM D-FPR-(cyclohexyl)ANSN. The rate of substrate hydrolysis was evaluated as described above. In a control experiment, the rate of substrate hydrolysis was evaluated for the same plasma sample, but in the absence of TF. The increased rate of substrate hydrolysis that was observed in the presence of TF (versus the control) was attributed to the activity of the factor VIIa/TF complex. Comparing the results to the

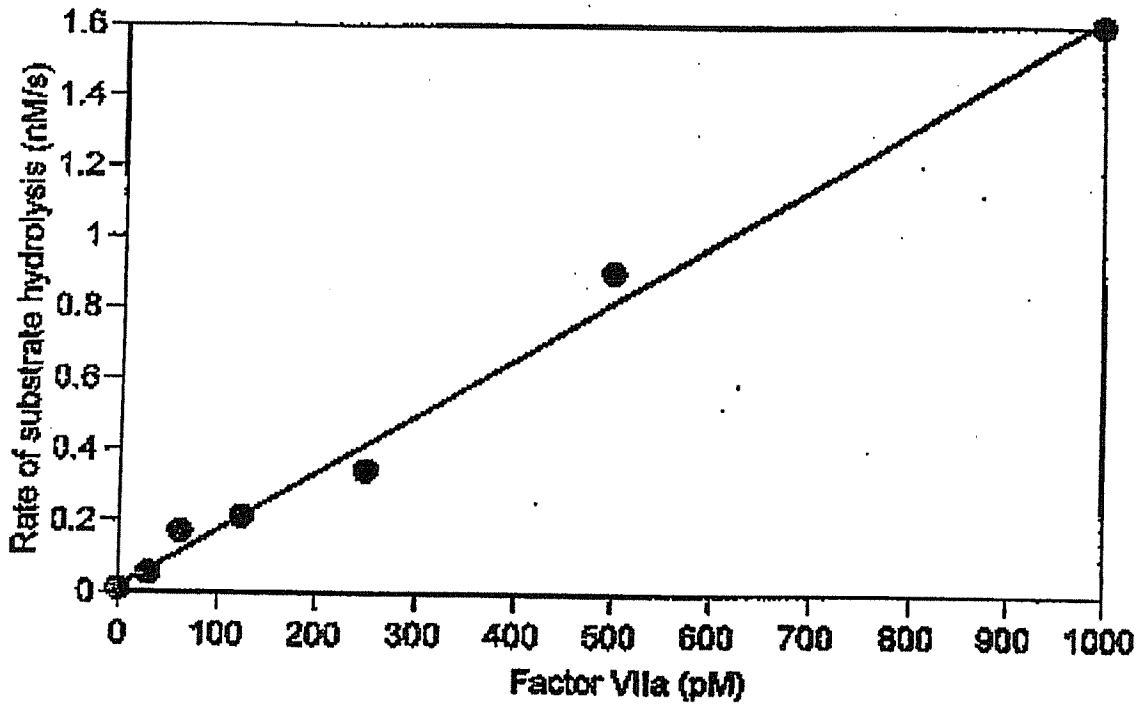


FIGURE 2: Calibration line for factor VIIa amidolytic activity.

calibration line in Figure 2, the plasma sample yielded a factor VIIa concentration of 102 pM. These preliminary results indicate that the factor VIIa concentration determined by this method is similar to that determined by the published clotting assay method [19].

Utilizing a similar approach, but in this case holding the factor VIIa concentration constant and varying TF concentration, an assay responsive to TF can be created. To construct a calibration curve for TF, varying concentrations of TF (0-500 pM) were incubated with 2 nM factor VIIa in HBS pH 7.4 containing 2 mM $CaCl_2$, for 10 min at room temperature, followed by the addition of 50 μM D-FPR-(n-butyl)ANSN. Substrate hydrolysis was monitored continuously for 5 min in a fluorometer at an excitation wavelength of 350 nm and an emission wavelength of 470 nm. Rates of hydrolysis were determined for each TF concentration tested. The rate of substrate hydrolysis by 2 nM factor VIIa alone was also measured and subtracted from rates observed in the presence of TF. A calibration line (hydrolysis rate vs TF concentration) was constructed (Figure 3). The rate of factor VIIa/TF-dependent substrate hydrolysis was linear over the range of TF concentrations tested.

In the next experiment, the same preparation of TF was relipidated into PCPS vesicles (composed of 75% phosphatidylcholine and 25% phosphatidylserine) as previously described[1]. Various preparations of relipidated TF (TF/PCPS: 250 pM/500 nM) were incubated with 2 nM factor VIIa in HBS, 2 mM $CaCl_2$ pH 7.4 for 10 min at room temperature followed by the addition of 50 μM D-FPR-(n-butyl)ANSN. Rates of substrate hydrolysis were determined and compared to the calibration curve above. Data from this assay (shown below) indicate that 35-40% of the TF added to the relipidation mixture is expressed as functional TF. These data indicate poor recovery of total TF activity following relipidation. Although a portion of the TF could have been

internalized into the vesicles, these recovery data support the need for a TF functional assay in the manufacture and quality control of thromboplastin reagents.

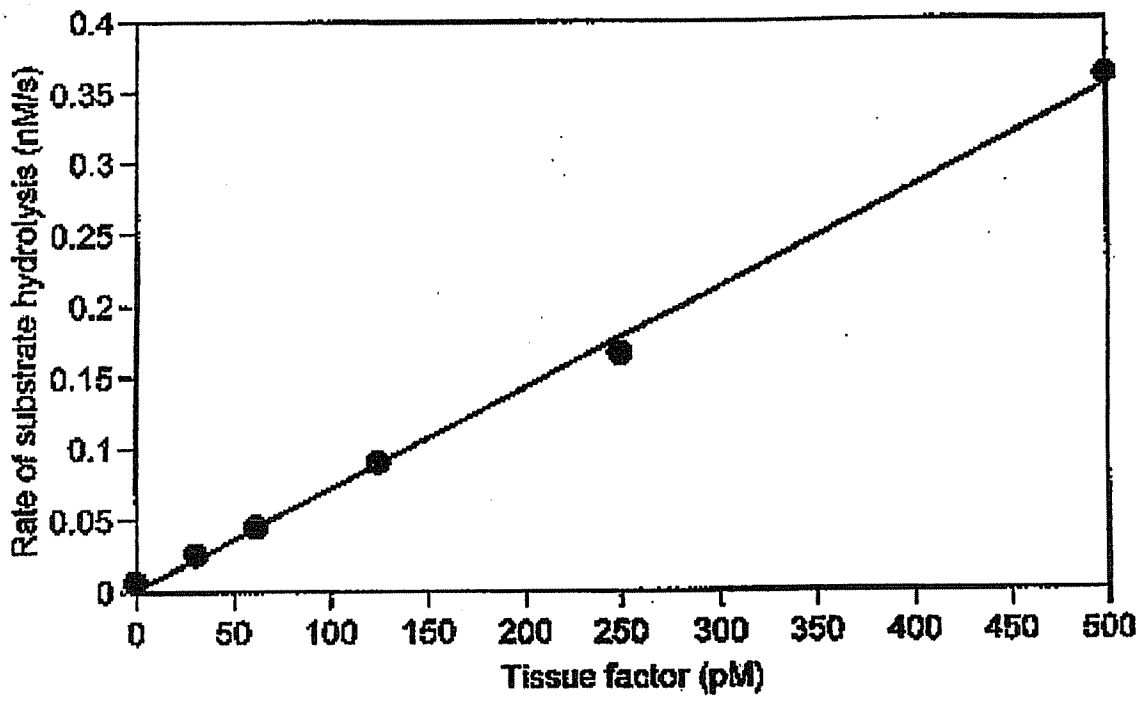


FIGURE 3: TF activity calibration line in the presence of 2 nM factor VIIa (fluorogenic assay).

TABLE 2: Recovery of active Tissue Factor following relipidation.
(Initial Tissue Factor Concentration = 250 pM)

Sample	Active TF found pM	%
Relipidation 1	97	39
Relipidation 2	88	35
Relipidation 3	101	40
Relipidation 4	93	37

In the same course of experiments, the lysate derived from frozen and thawed washed human platelets (2×10^8 platelets/ml in HBS, 2mM $CaCl_2$) was evaluated to determine if TF was present. The lysate was incubated with 2nM factor VIIa at room temperature for 10 min. Following the incubation, 50 µM D-FPR-(n-butyl)ANSN was added and the rate of substrate hydrolysis was determined over the course of 5 min. The rate of substrate hydrolysis was also measured in a

control experiment that was performed under identical conditions but in the absence of factor VIIa. The difference in the substrate hydrolysis rates were 0.06 nM/sec (the rate of substrate hydrolysis for 2 nM factor VIIa alone was negligible). By comparison to the calibration curve (Figure 3) the concentration of TF in the lysate was 90 pM. In a control experiment, purified TF was diluted to 90 pM in HBS, 2mM CaCl2 and was assayed under the same set of conditions yielding the same rate of substrate hydrolysis as the platelet lysate. When an anti-tissue factor monoclonal antibody was added to either the lysate or to the control experiment (done with purified TF), the rate of substrate hydrolysis was decreased by 50% thus confirming that the amidolytic activity was TF-related.

These preliminary data indicate that the ANSN-fluorogenic subtrates can be employed to detect TF and factor VIIa in relatively simple systems. This phase-I proposal is focused on extending this technology to demonstrate the feasibility of accurately determining TF and factor VIIa concentrations in more complex biological systems including, plasma, cells, cell lyzates and commercial thromboplastins.

D) RESEARCH DESIGN AND METHODS

This section will be organized according to the Specific Aims outlined in section A.

Reagents and Materials:

ANSN-fluorogenic substrates: Although there are a variety of different ANSN fluorogenic substrates that are useful for factor VIIa/TF we will focus our attention on those with either the ANSN-cyclohexyl or ANSN-n-butyl reporter groups. Preliminary data suggests that these two substrates will be the most useful for the intended applications. These substrates are standard catalog items of our own (catalog numbers SN-17a and SN-17c respectively; Haematologic Technologies, Inc.) and those required for this project will be manufactured in-house as needed.

Factor VIIa: Factor VIIa (also an HTI catalog item; catalog #HCVII-0020) will be prepared in-house by activating purified factor VII (10 µM in TBS, pH 7.4 containing 2mM $CaCl_2$) with factor Xa (10 nM) for 12 – 16 hrs at 4°C. Residual factor Xa will be removed by immunoaffinity adsorption and the remaining factor VIIa will be precipitated by the addition of ammonium sulfate to 75% saturation. The precipitated protein will be collected by centrifugation, formulated into 50% (vol/vol) glycerol water and stored at -20°C until needed. The required Factor VII will be purified from fresh frozen plasma according to published procedures which involve: a) barium citrate adsorption of citrated plasma; b) release from barium citrate by dissolution in the presence of EDTA; c) dialysis to remove EDTA and residual salts; and d) immunoaffinity chromatography over anti-factor VII-Sepharose.

Tissue Factor: Tissue factor is commercially available in both native and relipidated forms (American Diagnostica, Inc.; Greenwich, CT), however material for this project will come from in-house manufacturing operations in which recombinant TF is manufactured under contract by HTI for LifeScan, Inc. of Milpitas, CA. HTI has permission to use material for in-house R&D applications. At the present time, HTI is negotiating a license agreement with LifeScan that would allow HTI to manufacture and use TF for limited commercial applications.

PCPS Vesicles and Relipidated TF: Empty phospholipid vesicles and vesicles containing TF that are composed of 75% phosphatidylcholine (PC) and 25% phosphatidylserine (PS) will be prepared by the method of Mimms[43]. To prepare vesicles containing TF (relipidated TF) the

required amounts of PC and PS (dissolved in chloroform) are first dried to the walls of glass tube under a gentle stream of nitrogen. The lipids are then dissolved in HBS containing 100 mM octyl glucoside. TF which has also been formulated into HBS containing 100 mM octyl glucoside is added to achieve a final phospholipid to TF ratio of about 8000:1. The mixture is allowed to incubate at room temperature for 30 minutes, after which it is dialyzed against HBS to remove the detergent and allow the formation of phospholipid vesicles. The relipidated TF is stored at 4°C where it is stable for several days. Empty PCPS vesicles are prepared in the same manner but the TF is eliminated. PC and PS will be obtained from Avanti Polar Lipids, Alabaster, AL.

Human Plasma: Citrated human plasma which will be used for experimental purposes as well as for the manufacture of factor VII will be purchased from Seraplex, Inc., Duarte, CA. Seraplex, Inc. operates an FDA licensed plasma distribution facility. No donor identification information is available to HTI.

Human Platelets: Human platelets are routinely purchased by HTI through the BCA/hemerica network of blood banks and are washed prior to being used for manufacturing purposes. The same washed platelets will be used for assay development work involving human platelets and platelet lysates. No donor identification information is made available to HTI.

Miscellaneous reagents: Hirudin will be purchased from Calbiochem, La Jolla, CA. Fluorescent microtiter plates will be purchased from both Greiner Bio-One, Longwood, FL and Nalge Nunc International, Rochester, NY. Monoclonal antibodies against factor VIIa and TF are available in-house. All other chemicals and reagents will be purchased from approved HTI vendors and will be of the highest quality.

Specific Aim #1:

The preliminary data that was presented in section C was obtained using a standard fluorometer and experiments were therefore done in a cuvette format. We have performed a sufficient number of experiments with the ANSN-based fluorescent substrates in microtiter plate format using a Gemini fluorescent plate reader (manufactured by Molecular Devices, Sunnyvale CA) to confirm that the plate reader format will work for the projected applications. Adding to the complexity of assay development is the large number of different fluorescent assay plates that are available. We have experience using plates from both Nunc and Griener. Each company manufactures several different plates for which the nature of the plastic surface is different. The differences are often subtle and are generally related to surface charge and color. Knowing that different proteins react differently with each type of plate, we will include a survey of fluorescent microtiter plates in our preliminary experiments.

To establish basic assay parameters, assay conditions will be varied to find those that yield the highest precision, accuracy and detection limits. The effect of changing buffer pH and ionic strength, and the effect of adding or removing calcium ions will be examined. Factor VIIa will be titrated in the presence of saturating TF and visa-versa to determine the detection limits of the assay. Both fluorogenic substrates (SN-17a and Sn-17c) will be evaluated to determine which is optimal under each set of conditions. Polyethlyene glycol or Bovine Serum Albumin will be included in assay buffers if necessary to prevent loss of proteins due to adsorption.

With regard to the TF assay, both detergent solubilized and relipidated forms will be included in the assay development. Our preliminary data confirms our ability to assay both soluble and relipidated forms of TF.

To potentially enhance the sensitivity of the assays and to create format that will be less prone to matrix effects when the assay is applied to more complex biological systems, antibodies to factor VIIa or TF will be employed in immunocapture experiments. Murine anti-factor VII and murine anti-TF monoclonal antibodies will be immobilized onto high-binding fluorescent microtiter plates using conventional coating procedures (carbonate buffer, pH 9.6). Following immobilization step and removal of excess antibody, non-specific binding sites will be blocked with TBS containing 2% bovine serum albumin (BSA). The antibody plates will be used to capture the analytes from the sample matrix. Following a series of wash steps, the remaining assay components will be added. For the TF assay, saturating amounts of factor VIIa will be added followed by the addition of substrate. For the factor VIIa assay, saturating amounts of TF will be added followed by addition of the substrate. It should be noted that the rate of substrate hydrolysis is dependent upon formation of the factor VIIa/TF complex. Factor VIIa alone hydrolyzes the substrate at rate that are about 100-fold less that the factor VIIa/TF complex. For this reason, the excess enzyme (factor VIIa) in the TF assay will not substantially interfere and any signal that is from factor VIIa alone can be determined from a control sample and subtracted from the actual assay signal.

Upon completion of the work described in Specific Aim #1, we will have a good understanding of the assay limitations and of the effect of changing various assay parameters.

Specific Aim #2 and #3:

With assay conditions optimized for application to purified systems the assay will employed to examine more complex systems including synthetic and natural thromboplastins, plasma, platelets and platelet lysates. Challenges that are likely to be encountered will include matrix effects such as background scatter or fluorescence, non-factor VIIa-dependent substrate hydrolysis, influence of natural factor VIIa inhibitors, etc.

A limited amount of preliminary experience with measuring fluorescent signals directly from a plasma matrix indicates the presence of a small amount of background scatter or fluorescence that can interfere with the measure of substrate hydrolysis at low enzyme concentrations. Although we have confirmed that this interference can be minimized by photobleaching or charcoal adsorption, the effect that these treatments will have on the analytes is unknown. If the desired detection limits are not obtainable by direct measurement due to matrix effects, we will also explore the option of removing the sample from the matrix by immunoadsorption using antibody coated microtiter plates. For this application have a collection of factor VIIa and TF monoclonal and polyclonal antibodies available.

It is not anticipated that non-factor VIIa dependent substrate hydrolysis will pose a significant problem since we are able to sort out the factor VIIa signal due to the TF-dependent enhancement of this signal by almost 100 fold. Based upon this property we can subtract the non-TF dependent signal from the TF-dependent signal and thus yield a signal that is directly proportional to factor VIIa. The enzyme that is most likely to cause interference is thrombin and if necessary this signal can be blocked with a specific thrombin inhibitor such as hirudin. In addition, due to the specificity of our monoclonal antibodies, the immunoadsorption approach should effectively remove interfering enzymes from the assay system. It should also be noted that for plasma assays, EDTA will be included to prevent the generation of additional factor VIIa during assay procedures.

For factor VIIa, the primary biological material that we will focus on is plasma, however for TF we also plan to include platelets, platelet lysates and both natural and synthetic thromboplastins. Platelets will be assayed directly by making appropriate dilutions into HBS. Microtiter plates will be gently mixed during the assay procedure to ensure that a uniform suspension of the platelets is maintained. Platelet lysates will be prepared by a freeze/thaw cycle of platelets followed by centrifugation to remove platelet membranes. In addition, we will examine the supernatant of thrombin activated platelets after neutralization of the thrombin with hirudin.

In all assays, specificity will be confirmed by performing spike and recovery studies. These studies will be accomplished by spiking known amounts of purified protein (either factor VIIa or TF) into the biological mixtures being tested. Subsequent assay of this material will demonstrate the ability to measure the added analyte (i.e., recovery).

It is anticipated that the work performed during this phase I project period will demonstrate the feasibility of developing direct ultrasensitive fluorogenic-based assays for factor VIIa and TF that may be applied to the analysis of complex biological systems. Upon successful completion of the phase I project, we anticipate that a major R&D effort (phase II) will lead to the final development and validation of assays for commercialization.

E) HUMAN SUBJECTS

There will be no direct contact with human subjects. The only human materials to be used in this project are human plasma and human platelets. These materials will be purchased from FDA licensed companies/organizations (as outlined in section "D") that sell these materials for manufacturing purposes. No donor information is available to HTI.

F) VERTEBRATE ANIMALS

There will be no vertebrate animals used in this project.

G) LITERATURE CITED

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H) CONSORTIUM/CONTRACTUAL ARRANGEMENTS

None.

I) LETTERS OF SUPPORT

Letters of support from Dr. Saulius Butenas and Dr. Kenneth G. Mann are attached. These individuals will serve as consultants for this project.

J) PRODUCT DEVELOPMENT PLAN

Does not apply to this Phase I SBIR application.

The UNIVERSITY of VERMONT
COLLEGE OF MEDICINE
DEPARTMENT OF BIOCHEMISTRY

July 31, 2002

Richard J. Jenny, Ph.D.
President and Scientific Director
Haematologic Technologies, Inc.
57 River Road, Unit 1021
Essex Junction, VT 05452

Dear Rick:

I have reviewed your SBIR proposal that is entitled "Fluorogenic Assays for Factor VIIa and Tissue Factor" and am very enthusiastic about the potential to work with you as a consultant on this project. As you are aware, I have a tremendous amount of experience with the ANSN family of fluorogenic substrates with regard to both preparation and application. My studies have involved the use of these substrates for the elucidation of mechanisms involving factor VIIa and tissue factor and believe that your proposal is a nice extension of my research endeavors. As a consultant, I would be available both as an advisor and as an assistant to help you with certain elements of this project. I wish you the best of luck in securing the necessary funding for this project.

Sincerely,

Saulius Butenas, Ph.D.
Research Associate Professor

Health Science Complex, Given Building, 89 Beaumont Avenue, Burlington, VT 05405-0068
Telephone: (802) 656-2220, Fax: (802) 862-8229

The UNIVERSITY of VERMONT
COLLEGE OF MEDICINE
DEPARTMENT OF BIOCHEMISTRY

July 31, 2002

Richard J. Jenny, Ph.D.
President and Scientific Director
Haematologic Technologies, Inc.
57 River Road, Unit 1021
Essex Junction, VT 05452

Dear Rick:

I am writing to express my willingness and enthusiasm to act as a consultant on your project entitled "Fluorogenic Assays for Factor VIIa and Tissue Factor". A robust assay for these proteins that can be applied to complex biological systems does not exist. I believe that the assays you are proposing to develop are essential tools that will have both research and diagnostic applications. I will be available to consult with you on any issues that pertain to this project.

Sincerely,

Kenneth G. Mann, Ph.D.
Professor and Chair

Health Science Complex, Given Building, 89 Beaumont Avenue, Burlington, VT 05405-0068
Telephone: (802) 656-2220, Fax. (802) 862-8229

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

☒ NEW application. *(This application is being submitted to the PHS for the first time.)*
☒ SBIR Phase I ☐ SBIR Phase II: SBIR Phase I Grant No. _____ ☐ SBIR Fast Track
☐ STTR Phase I ☐ STTR Phase II: STTR Phase I Grant No. _____ ☐ STTR Fast Track

☐ REVISION of application number: _____
(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

☐ COMPETING CONTINUATION of grant number: _____
(This application is to extend a funded grant beyond its current project period.)

☐ SUPPLEMENT to grant number: _____
(This application is for additional funds to supplement a currently funded grant.)

INVENTIONS AND PATENTS
(Competing continuation appl. and Phase II only)
☐ No ☐ Previously reported
☐ Yes. If "Yes," ☐ Not previously reported

☐ CHANGE of principal investigator/program director.
Name of former principal investigator/program director: _____

☐ FOREIGN application or significant foreign component.

1. PROGRAM INCOME ***(See Instructions.)***
All applications must indicate whether program income is anticipated during the period(s) for which grant support is request. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)

2. ASSURANCES/CERTIFICATIONS ***(See instructions.)***
The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/certifications are provided in Section III. If unable to certify compliance, where applicable, provide an explanation and place it after this page.

•Human Subjects; •Research Using Human Embryonic Stem Cells• •Research on Transplantation of Human Fetal Tissue •Women and Minority Inclusion Policy •Inclusion of Children Policy• Vertebrate Animals• •Debarment and Suspension; •Drug- Free Workplace *(applicable to new [Type 1] or revised [Type 1] applications only)*; •Lobbying; •Non-Delinquency on Federal Debt; •Research Misconduct; •Civil Rights (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age Discrimination (Form HHS 680 or HHS 690); •Recombinant DNA and Human Gene Transfer Research; •Financial Conflict of Interest (except Phase I SBIR/STTR) •STTR ONLY: Certification of Research Institution Participation.

3. FACILITIES AND ADMINSTRATIVE COSTS (F&A)/ INDIRECT COSTS. See specific instructions.

☐ DHHS Agreement dated: _____ ☐ No Facilities And Administrative Costs Requested.

☐ DHHS Agreement being negotiated with _____ Regional Office.

☐ No DHHS Agreement, but rate established with _____ Date _____

CALCULATION* [JG7]*(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information.)*

a. Initial budget period:	Amount of base $		x Rate applied		% = F&A costs	$	
b. 02 year	Amount of base $		x Rate applied		% = F&A costs	$	
c. 03 year	Amount of base $		x Rate applied		% = F&A costs	$	
d. 04 year	Amount of base $		x Rate applied		% = F&A costs	$	
e. 05 year	Amount of base $		x Rate applied		% = F&A costs	$	
					TOTAL F&A Costs	$	

*Check appropriate box(es):

☐ Salary and wages base ☐ Modified total direct cost base ☐ Other base *(Explain)*
☐ Off-site, other special rate, or more than one rate involved *(Explain)*

Explanation *(Attach separate sheet, if necessary.)*:

4. SMOKE-FREE WORKPLACE ☒ Yes ☐ No *(The response to this question has no impact on the review or funding of this application.)*